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PRINCIPAL INVESTIGATOR: Luciane R.Cavalli PH, D.

CONTRACTING ORGANIZATION: Georgetown University
Washington DC 20057-1411

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14. ABSTRACT The sentinel lymph node (SLN) is the first node in the mammary gland to harbor malignant cells in breast tumors with metastasis, and SLN positivity is an indication for axillary lymph node dissection. The purpose of our study is to identify specific genetic alterations using array-CGH in the metastatic sentinel lymph node lesions, in comparison to the ones observed in the corresponding primary tumors from patients with breast cancer. We believe that the characterization of genetic alterations at the SLN site is a logical step to define the cytogenetic evolution of primary tumors to a metastatic state, and may represent the initial genetic events that occur in the early metastatic process, before distant metastasis occur. Ultimately these alterations can be used as molecular markers that can help in the reduction or elimination of the need for invasive surgical procedures, such as axillary dissection, in the management of breast cancer patients.				
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DETECTION OF GENETIC ALTERATIONS IN BREAST SENTINEL LYMPH NODE BY ARRAY-CGH

P.I. Luciane R.Cavalli, PhD

INTRODUCTION

Axillary lymph node status remains the single most important prognostic marker in breast cancer and a key component of its staging system. The sentinel lymph node (SLN) is considered the first node to harbor malignant cells in breast tumors with metastasis (Veronesi et al, 2001). Currently, the decision to proceed with a complete axillary lymph node dissection is based on the SLN positivity (Schwartz et al, 2002; Kim et al, 2005; Lyman et al, 2005). In early stage breast cancer, several studies have shown that when the SLN is negative, future recurrence in the axillary lymph nodes is rare (Arpana et al, 2004; Swenson et al, 2005). Therefore the histopathologic evaluation of the SLN in an effort to identify even the smallest metastatic foci at this site, at the time of the initial surgery, is critical. The decision to proceed with a complete axillary lymph node dissection depends on the detection of tumor cells in the SLN. Several studies, mostly based on RT-PCR assays, have identified mRNA markers, such as *ERBB-2*, *TP53*, *VEGF*, *h-MAM*, to have significant prognostic potential in breast cancer with respect to being associated with axillary lymph node metastasis (Backus et al, 2005; Cannone et al, 2006; Nathanson et al, 2006; Nissan et al, 2006). However, and despite the advances in identifying some promising tumor markers in the SLN, currently, none of these “potential” markers is used, neither individually nor in combination, to influence the decision for a complete axillary node dissection, a decision solely dependent on the presence or absence of tumor cells in the SLN as determined by histopathologic evaluation (Hughes et al, 2006).

In an effort to predict metastasis, several studies assessing gene expression in breast tumors have been published (Perou et al, 2000; Sorlie et 2001; West et al, 2001; Van de Vijver et al, 2002; Van't Veer et al, 2003; Weigelt et al, 2003). However none of these studies have been based on the SLN status, but rather on more distant axillary lymph node metastasis. In addition, very few studies have been performed in paired primary tumors and their corresponding metastatic lesions in the same patient. On the other hand, assessment of copy number changes in primary breast tumors and in their corresponding metastatic lesions, is also critical, as it can define whether metastatic lesions exhibit the same type of genetic alterations found in primary tumors, and whether there are specific types of alterations in the primary tumors that make metastasis more likely to occur. We previously reported the only study where genetic and epigenetic alterations were evaluated in SLN metastatic lesions and compared these alterations to the ones in the corresponding primary tumors in the same patient (Cavalli et al, 2003). Although our study was a small pilot study consisting of six patients, where we evaluated primary and sentinel lymph node lesions using comparative genomic hybridization (CGH) and methylation assays, we observed that, in general, in each separate case the number and complexity of the alterations found in the two groups were similar, and in every case there were at least two chromosomal abnormalities common to both the primary tumors and the SLN metastatic lesions. In addition, no significant difference in the total number of chromosomal changes was observed between the two groups.

Our long-range goal is to identify new predictive markers in order to augment the current criteria used to assess breast cancer prognosis. The aim of the current project is to compare genomic alterations between primary breast tumors and their corresponding sentinel lymph node metastatic lesions in order to identify a subset of relevant genetic alterations associated with metastasis.

We hypothesize that:

- a) Genetic alterations in the sentinel lymph node lesions are likely to represent early and significant changes in the process of metastasis and are likely to occur before metastasis takes place.
- b) Characterization of such changes may aid in defining the cytogenetic evolution of primary tumors to a metastatic state, and therefore, in discovering new prognostic markers.

The identification of this subset of genes is critical to understand the progression of primary tumors to an early metastatic disease and ultimately can be used to predict breast cancer metastasis and identify individuals at higher risk of developing axillary lymph node metastasis. For this reason, the identification of genetic predictors of SLN metastasis may improve the prognostic value of the SLN biopsy in breast cancer patients.

There is a critical need for the development of more sensitive methods to identify new prognostic markers to improve breast cancer staging at the time of diagnosis, and predict disease relapse in breast cancer patients. Towards this end, we have conducted the current study in order to analyze changes in the DNA copy number in primary tumors and their corresponding sentinel lymph node metastatic lesions from the same patients, using both conventional and array-CGH methodology. The aim is to compare genomic alterations between the two types of lesions and identify a subset of relevant genetic alterations associated with metastasis.

CGH analysis of tumors is a well-established method to ascertain DNA copy number changes. Since it was first described in Science in 1992 by Kallioniemi *et al*, it has proven its usefulness as a highly informative approach for genome-wide screening of tumors. Its highest impact continues to be in its application to studies of formalin fixed paraffin embedded (FFPE) tumors. Indeed, the vast majority of archived tumor specimens available for use in research from tumor banks and repositories around the world, are formalin fixed paraffin embedded.

Although CGH continues to play a significant role in improving our knowledge about tumor biology in various ways including understanding genetic events controlling tumor initiation and progression and identifying diagnostic/prognostic molecular markers, its main limitation is its relatively limited resolution (about 5-10 Mb). Recently, in order to improve the resolution of the CGH technology, several array-CGH approaches have been developed in order to provide higher-resolution mapping of the gains and losses in the tumor genome. Accurate definition of copy number changes in primary breast tumors allows a better assessment of the aberrations and a more precise definition of the genomic changes that are present in these lesions. Array platforms that are currently in use for CGH analysis include, large insert clones, i.e. Bacterial Artificial Chromosome (BAC)-arrays (Resolution 100 Kb), cDNA-arrays

(Resolution 2 Kb), and oligonucleotide (Oligo)-arrays, which have a significantly higher resolution (around 0.06 Kb), then the first two methods. Furthermore, it is difficult to control the specificity of the hybridization to the complex sequences that are present in each feature of the first two types of arrays (BAC array and cDNA-arrays). Because of the higher resolution of oligo-arrays and the likelihood of a better specificity then other array methods, we have optimized a method to assess formalin fixed, paraffin embedded (FFPE) microdissected tumor tissues using oligo array-CGH (Agilent Platform) (Agilent Technologies, Palo Alto, CA).

BODY

The main purpose of this project is to identify a subset of specific genetic alterations in breast tumors that may be used to predict metastasis. To achieve this goal, we evaluated primary breast tumors and their corresponding metastatic sentinel lymph node (SLN) lesions in order to detect DNA copy number gains and losses using comparative genomic hybridization (CGH). Findings were compared between each primary tumor and its SLN lesion, as well as between the two groups (all primary tumors vs. all SLN lesions). The frequency and type of the genetic alterations were identified and analyzed. *Our findings suggest that, despite the known clonal divergence and genetic heterogeneity that characterize the metastatic process, there is an identifiable pattern of genetic changes that may control the process, and therefore a subset of genetic alterations that could predict the metastatic potential of primary breast tumors can be identified.*

Report of the accomplishments

1. Specimen Accrual

We completed the accrual of thirty patients with invasive breast cancer and SLN involvement at the time of diagnosis. These patients were selected from the Pathology Department at the Hospital Nossa Senhora das Gracas, Paraná, Brazil using the following selection criteria:

- Breast cancer patients with long term follow up (>5 years).
- Sentinel lymph node positive.
- Paired tissues available from primary tumors and corresponding sentinel lymph nodes.

The samples were collected with the patients' informed consent and IRB approval of the Hospital Nossa Senhora das Gracas and CONEP (National Review Board of Ethics in Research-Brazil) and Georgetown University. The samples were received with no patient's identifiers. All relevant clinical information on the patients was made available to us, including: clinical follow for each patient for over 5 years from the time of diagnosis, Stage and Grade of the disease, hormonal receptor (ER/PR) and *HER2/NEU* status, time of recurrence, type of treatment, disease free survival time, and survival, are available and updated as necessary (i.e. when change occurs).

2. Histopathological review and isolation of tumor cells

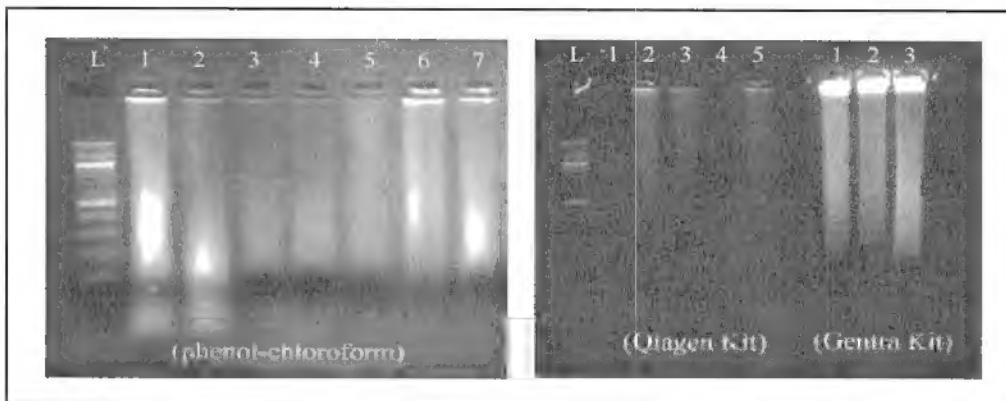
In each case, an H&E section was reviewed by a pathologist with expertise in breast histopathology at Georgetown University, in order to confirm the presence of tumor tissues in the paraffin block under investigation. The pathologist delineated and marked the areas with tumor tissues on the H&E slide in order to facilitate their identification and isolation from a consecutive paraffin section for CGH analysis. Tumor cells were isolated using either Laser Capture Microdissection (LCM) or gross needle microdissection (depending on the size of the lesions), using a well-established protocol in our lab, which was, published earlier (Cavalli et al, 2004). The tissue microdissection step was performed to ensure that tumor cells were appropriately selected and to reduce the “contamination” of the tumor cells with normal or stromal cells, ensuring that the findings accurately reflect alterations of the tumor genome.

3. DNA preparation from formalin fixed, paraffin embedded (FFPE) samples

We have optimized the methods to prepare DNA from FFPE samples that can be used for subsequent CGH analysis. It is very well established that the quality and quantity of the DNA obtained from formalin fixed, paraffin embedded tissues is highly variable, and nearly all cases show DNA degradation to various degrees. Depending on the assay, the extent of DNA degradation directly correlates with the quality and success rates of the assay. CGH analysis of tumors is no exception. Based on our long-term experience with CGH analysis and on that of others in the field, the quality and reliability of the CGH data is directly correlated with the quality of the DNA to be tested. While this is true for both chromosomal CGH and array-CGH studies, the latter are much less tolerant to DNA degradation. DNA quality is the single most important variable controlling the success of array-CGH analysis.

We dedicated a significant amount of effort to evaluate several DNA isolation protocols/kits and compare the quality and quantity of DNA obtained from the tumor tissues following microdissection and the ability to use the prepared DNA for CGH studies. Three protocols were found to be satisfactory in providing DNA of acceptable quality and quantity to be subsequently used for CGH analysis, namely, a phenol/chloroform protocol, a kit manufactured by Gentra (Genomic DNA purification kit-Puregene DNA purification system-Gentra Systems, Minneapolis, MN, USA), and a kit manufactured by Qiagen (DNeasy blood and tissue kit-Qiagen Inc., Valencia, CA, USA.). **Figure 1** shows images of Agarose gels for DNA extracted using either of the 3 approaches. While all three protocols provided DNA which was adequate for chromosomal CGH analysis, we selected the kit manufactured by Gentra for DNA preparation for array-CGH analysis, because it showed slight but detectable advantage over the other two DNA extraction approaches and was more commonly used by other researchers using the same array platform as ourselves (Agilent Arrays).

Figure 1: DNA isolation of FFPE material from primary breast tumors and sentinel lymph node samples using different methodologies. The numbers indicate the sample numbers and L shows the DNA ladder X174. In the DNA isolation using the Qiagen and Gentra kits, samples 1, 2 and 3 were from the same case, and were isolated simultaneously.



4. Analysis of DNA copy number changes by CGH

We successfully completed the analysis of 20 pairs of primary breast tumors and corresponding sentinel lymph node metastatic lesions (40 tumor specimens) using CGH, from the 30 patients that were accrued. In few cases, we had results either from the primary tumor alone or from the SLN lesion alone but not from the pair. Since our objective is to compare the findings between each primary tumor and its SLN lesion, these cases were not included in the analysis and will be included in the future, once the corresponding lesion is successfully evaluated by CGH. Based on the data obtained from this study we observed that:

- i) Chromosomal abnormalities were detected in all 20 cases, in both the primary tumors and the SLN lesions (40 tumor specimens).
- ii) Despite the diversity in the alterations detected in each case, there were common alterations shared by both the primary tumors and their corresponding metastatic SLN lesions. 17 pairs of primary tumors and corresponding SLN lesions, shared at least 2 alterations while 5 pairs: cases # 1, 3, 7, 13 and 16 shared almost all the aberrations. **Table 1** summarizes the aberrations shared between each primary tumor and its corresponding metastatic SLN lesion.
- iii) A subset of the detected aberrations was commonly observed in both groups, occurring in 30-60% of the cases. These included: gain of +1p13-pter, +1q, +12q23-qter, +16, +19, +20 and loss of: 2q22-q34, 6q13-q23, 13q13-q32, Xq21-q26. **Table 2** summarizes the most common abnormalities observed and their frequencies in each group.
- iv) The above alterations were also the most commonly observed in the whole set.

- v) Most interestingly, we identified a subset of chromosomal alteration which were more commonly detected in either the SLN lesions or the primary tumors, specifically: Loss of 6q (whole arm or a segment of it) and gain of chromosome 20 (whole chromosome) were more frequently observed in the SLN group, whereas gain of 12q and 20q were more frequently seen in the primary tumors. Gain of 6p was detected only in the SLN lesions in 25% of the cases.
- vi) A representative profile of a primary tumor and its corresponding sentinel lymph node metastatic lesion from the same case is shown in **Figure 2**. Note some of the most common DNA copy number changes observed in this case: specifically: +1p13-pter, -6q13-q23, -13q13-q32, +19, +20q13, among others.

Table 1: Summary of the aberrations shared between each primary tumor and its corresponding metastatic SLN lesion.

Case #	Common DNA copy number changes in PT and SLN lesions
1	+1p32-pter, -2q22-q36, -4q, -6q13-q23, ++9q21-qter -13q13-q32 +16, +17, +19
2	-13q13-q32, +16p
3	+1q22-qter, +2q24-qter, +8q23-qter, +12q23-qter, -13q13-q32, -15q23-qter, -16q13-qter
4	+1p21-q24, +12q23-qter, +16
5	+1q22-qter, -1p21-pter, -6q13-q23, ++11p15-q21, +16, +20
6	+11p15-q21, -13q13-q32,
7	+1p33-pter, -1p31-p21, +1q33-pter, -2q22-q34, -5, +12q24.1-qter, +15q22-q23, +16, +17, +20, -Xq21-qter
8	+1p32-pter, -6q13-q23, +16p, +17, ++19, ++20
9	-6q13-q23, -18q21-qter, +Xq13-q22
11	+1p31-pter
12	-6q, +19, -X
14	+1p32-pter, +19p
16	-2q22-q34, -5q12-q22, -6q, +8p, +9q31-qter, -11q14-q22, +11p15-pter, ++12q23-qter, -13q13-q32, +14q24-qter, +16, +17, -18q21-qter, +19, ++20q, -Xq21-q26,
17	-2q13-q31, +16p, +18p, +19p, +20p
18	+1p32-pter, +17p, +19, +20,
19	+17, ++19,
20	+19, +20p

-: Loss

+: Gain

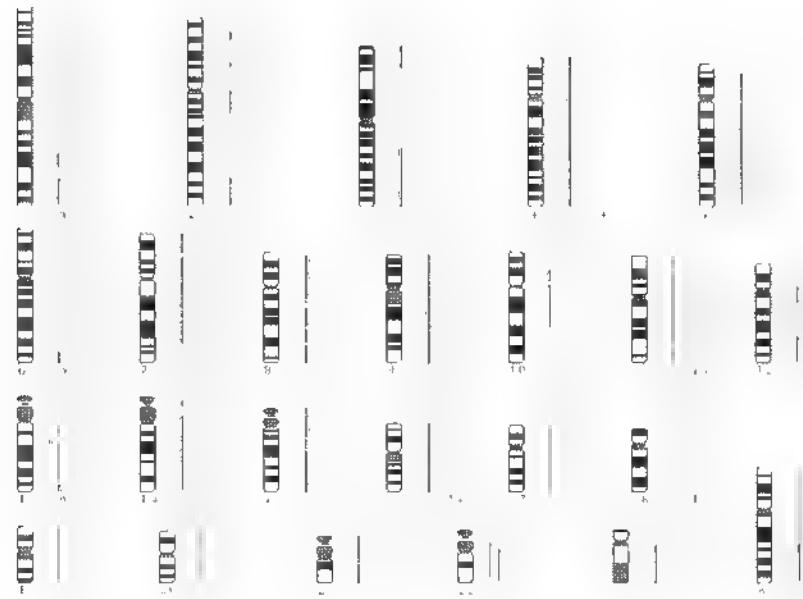
++: Amplification

Table 2: Summary of the most common abnormalities observed and their frequencies in each group.

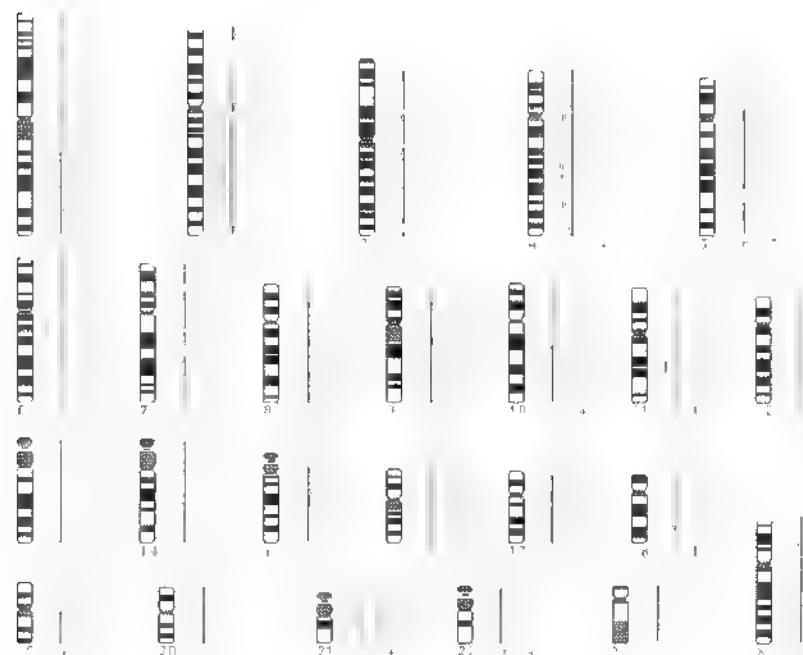
DNA copy number change	Primary Tumors	SLN lesions
+1p13-pter	10 (50%)	6 (30%)
+1q	7 (35%)	7 (35%)
-2q22-q34	7 (35%)	8 (40%)
-6q13-q23	10 (50%)	11 (55%)
+12q23-qter	8 (40%)	5 (25%)
-13q13-q32	11 (55%)	8 (40%)
+16	8 (40%)	8 (40%)
+19	12 (60%)	12 (60%)
+20	14 (70%)	9 (45%)
- Xq21-q26	8 (40%)	7 (35%)

Figure 2. CGH profile of the primary breast tumor (A) and its corresponding sentinel lymph node metastatic lesion (B) from case #1. The vertical lines on the right side of the ideograms reflect different values of the fluorescence ratio between the test and the normal DNA. The values are 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 from left to right. Ratios of 1.25 or higher reflect gains whereas ratios of 0.75 or lower reflect losses. N is the number of chromosomes used to generate each ratio profile.

A



B



5. Analysis of DNA copy number changes by array-CGH

We have optimized the conditions to perform array CGH analysis of DNA obtained from formalin fixed, paraffin embedded (FFPE) microdissected tumor tissues using oligonucleotide arrays (Agilent Platform).

Because of the higher resolution of oligo-arrays and the likelihood of a better specificity than other array methods, we set out to develop an approach to analyze DNA obtained from formalin fixed, paraffin embedded (FFPE) microdissected tumor tissues using oligo-arrays (Agilent Platform) (Agilent Technologies, Palo Alto, CA). This platform consists of 44,000 probes of 60-mer arrayed oligonucleotides, covering the whole genome. We chose this approach in order to refine the findings obtained through chromosomal CGH analysis. Specifically: refine the breakpoints of the regions already identified as gained or lost in the tumor genome, and detect additional changes that were missed by the initial CGH analysis that we completed.

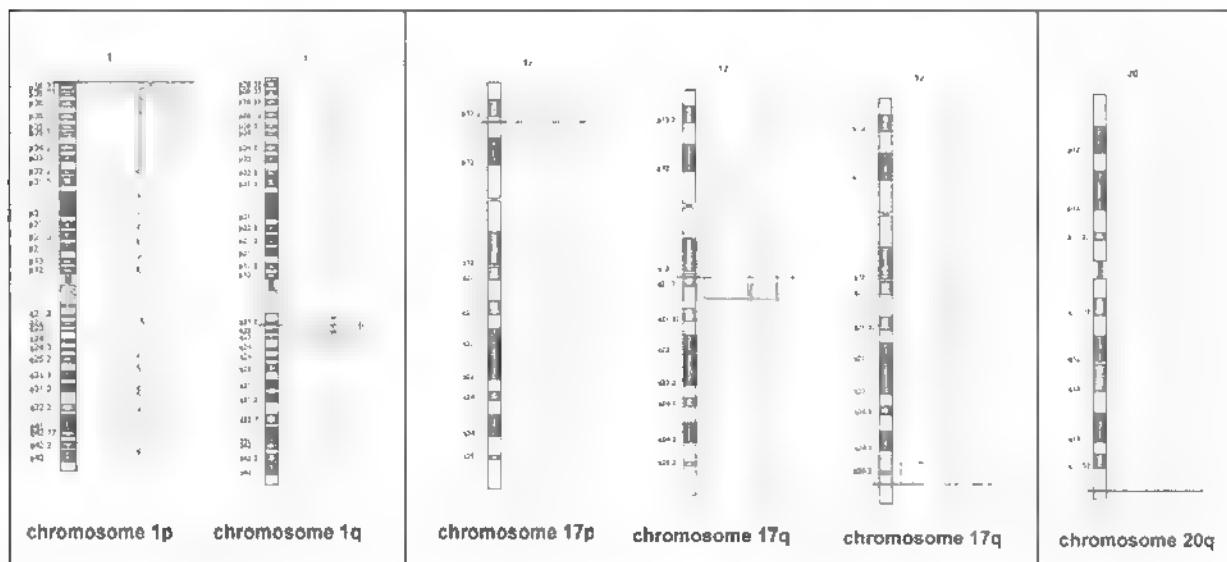
While there has been few reported studies using oligo-array CGH in the past two years since it was initially reported (Barrett et al. 2004), all reported studies used either cell lines or frozen material as a source of tumor cells. No reported studies described the use of formalin fixed paraffin embedded tissues as the source. In this project, our approach was to optimize a method to successfully use FFPE tissues for oligo-array analysis. This part of the project required major effort because the array CGH method was published only two years ago, and so far, no studies have been reported using FFPE material. We encountered several unexpected difficulties to establish the methods. As described in the previous sections, (3- and 4- above), we tested several protocols for DNA preparation and selected the one developed by Gentra.

We evaluated different protocols for DNA labeling and showed that nick translation labeling was adequate for conventional CGH while random primed labeling (Bioprime labeling kit - Invitrogen) was the preferred method for array-CGH analysis. We also assessed the possibility of introducing a whole genome amplification step prior to DNA labeling in order to increase the amount of DNA available for labeling. We tested two different protocols using two different kits from Qiagen: Qiagen REPLI-g Mini kit, and REPLI-g Damage DNA Field test kit. The first one is commercially available and the other is still under development (we served as a beta site for the later kit). Our experiments showed that this amplification step introduced additional biases to the procedure without significantly improving the success rate of the labeling procedure. For this reason, we found this step to be unnecessary.

To optimize the oligo array CGH methodology, we have initiated an active collaboration with Dr. Thomas Ried and his group at the National Cancer Institute/NIH. Dr. Ried is an international authority in molecular cytogenetic analysis of tumors. His lab has established oligo-array CGH analysis using the Agilent platform to evaluate tumor cell lines. Because of the expertise of Dr. Ried's group in oligo array-CGH analysis and of our mutual interest to develop the method for FFPE samples, we started this collaboration. We also worked closely with Agilent scientific team as consultants, to overcome many of the problems associated with the quality of the hybridization and the intensity of the signal. We performed several experiments under multiple conditions that lead to the development of a robust protocol applicable for FFPE specimens. Data from the evaluation of three specimens is shown in **Figures 3-7**. Our results showed that the chromosomal gains and losses detected by the oligo array CGH analysis were similar to the ones observed using chromosomal CGH. However, oligo array CGH allowed the definition of specific genes which are altered. This is a major advantage of the oligo array CGH methodology.

Based on the work that we completed, we conclude that conventional CGH analysis of the specimens prior to array CGH analysis is a very crucial step and very informative; it is arguably the best way to assess the quality of DNA available for array-CGH analysis. Although conventional CGH analysis is more “tolerant” to the quality of the DNA as compared to array CGH analysis, cases with inadequate DNA for conventional CGH analysis (i.e. DNA which is too degraded), generally fail in array analysis. Therefore testing the samples by conventional CGH prior to array-CGH analysis, allows the selection of samples with “good” quality DNA to test with arrays (to save on the cost of arrays). In addition, and since the oligo-array technology was recently described, this allows the opportunity to compare the data generated by these two methods.

Figure 3: Oligo array-CGH profiles of three different chromosomes 1, 17 and 20, obtained from the analysis of a primary breast primary tumor using the Agilent array-CGH platform (in the blue squares are the chromosomal regions that show the DNA copy number alterations).



In the chromosome regions shown to be altered above, several genes with importance to breast cancer tumorigenesis are localized, and are shown in our samples, to present DNA copy number alterations, such as amplifications and deletions.

As an example, genes that are mapped at the 17q21 region (Figure 4), such as: *HER-2*, *DLX* (*DLX-3* and *DLX-4*) and *TOPA2* genes. The *HER2/NEU* gene is amplified in 20-30% of invasive breast carcinomas (Nicolini et al, 2006), and is associated with tumors that present aggressive features, such as high proliferation rate, high frequency of aneuploidism, shorter disease free-survival and overall survival (Slamon et al, 1987). Metastatic breast cancers that present *HER2* overexpression and/or gene amplification, have been shown to respond well to the chemotherapy drug, trastuzumab (an monoclonal antibody against the extracellular domain of the *HER-2* protein) (Moulder et al, 2001). Interestingly, the *DLX* genes, have been shown to be co-

amplified with *HER-2* gene (Man et al, 2005). Members of the distal-less (DLX) family of homeobox genes have been implicated in breast tumorigenesis (Fu et al, 2003) and a recent study have shown that DLX-4 mRNA was detectable in 80% of infiltrating ductal carcinoma and its expression was found to correlate with breast cancer progression and invasion (Man et al, 2005). In our cases, the samples that presented amplification of the *HER-2* gene (Figure 5A) also present amplification of the *DLX* genes (see Figure 5B). We confirmed the findings of these two genes detected to be amplified using FISH analysis. (Figure 6). The *TOP2A* gene, topoisomerase II α , has been shown to be co-amplified or deleted in tumors with *HER-2* amplification (Jacobson et al, 2004; Bouchalova et al, 2006). Co-amplification of *TOP2A* has been suggested to play an important role in facilitating the response to anthracyclines, which targets *TOP2A* (Coon et al, 2002). In our samples we did not observed co-amplification between these two genes (Figures 5 and 7). Analysis of an additional number of cases are needed to confirm if there is a co-amplification or independent mechanism of amplification between these genes in breast cancer.

Figure 4: Detailed analysis of the 17q21 chromosome region (green bar) showing several genes amplified (red dots) and deleted (green dots) in a sample of primary breast tumor analyzed. This region was chosen because it harbors genes that are well known to be amplified in breast cancer such as: *HER2/NEU* and *BP1 (DLX4)*.

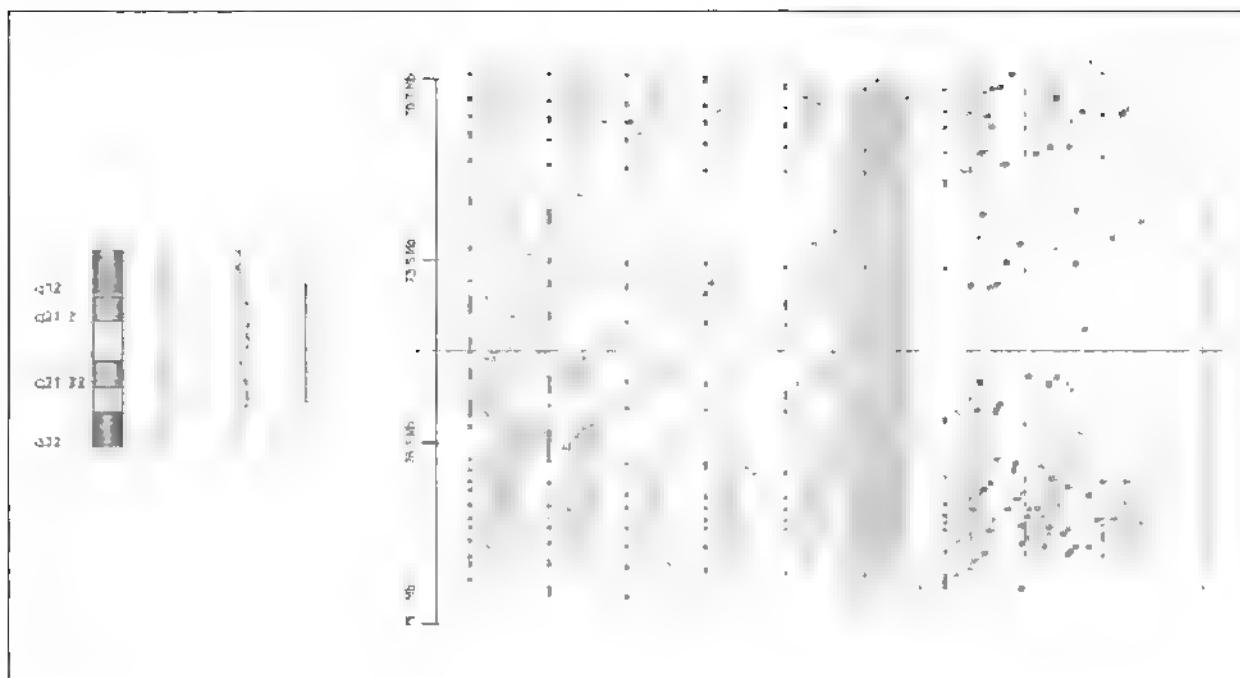


Figure 5: A detailed image of the 17q21 region showing the amplification (red dot) of the *HER2/NEU* (A) and *DLX-3* and *BPI* (*DLX-4*) genes (B) and deletion (green dot) of the *TOP2A* gene (C).

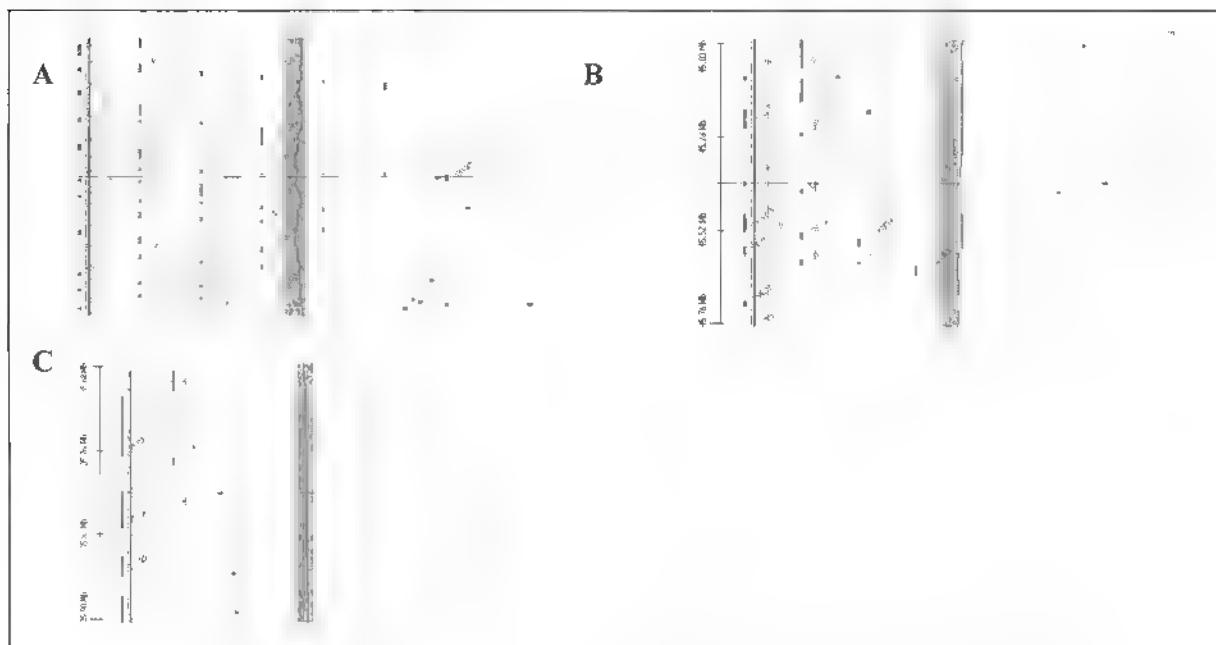


Figure 6: Confirmation of the findings by oligo array-CGH analysis using FISH analysis. As shown in Figure 5 above, oligo array-CGH analysis of this case showed amplification of the *HER2/NEU* and *BPI* (*DLX-4*) genes. FISH analysis using probes for *DLX4* and *HER2/NEU* genes shown in this figure, confirmed the amplification of both in that case.

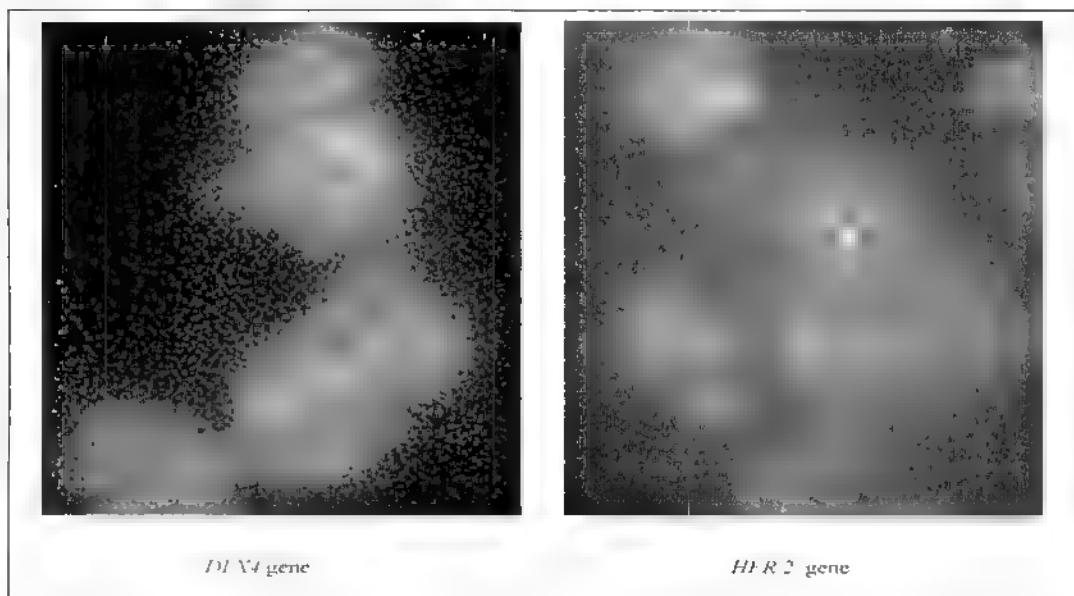
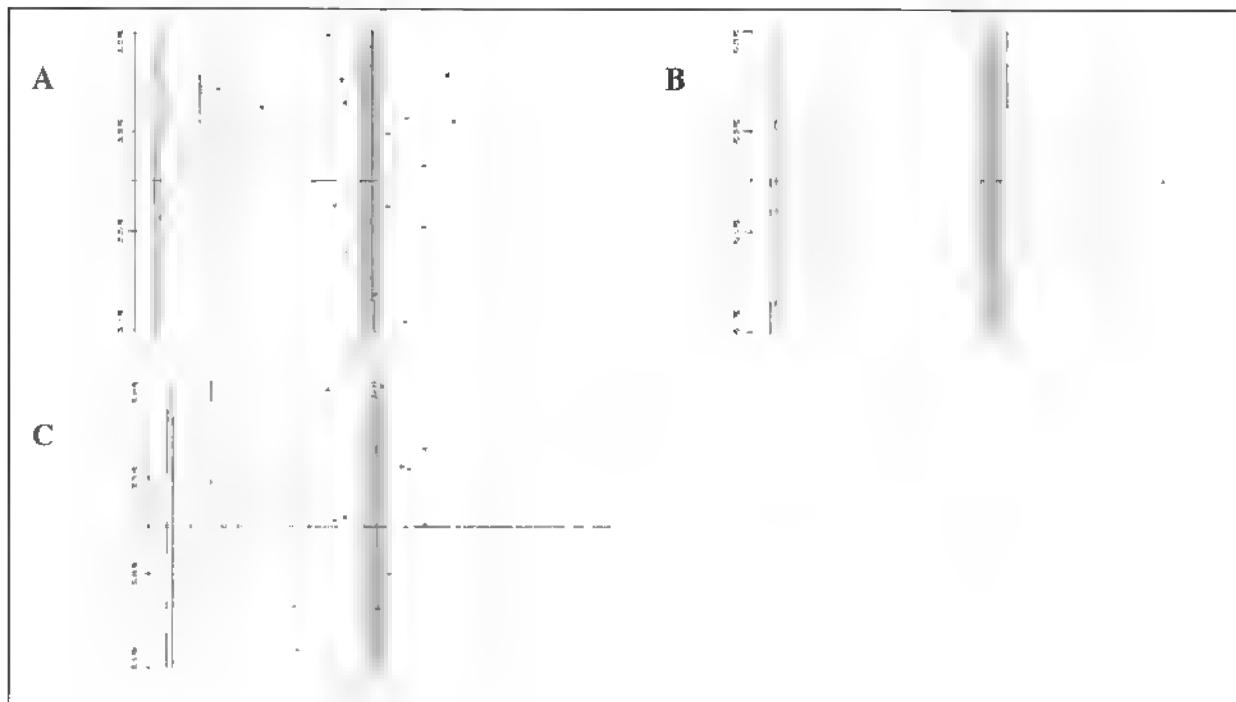


Figure 7: A detailed image of the 17q21 region from another primary tumor showing loss of DNA copy number (green dot) of the *HER2/NEU* (A) and amplification (red dots) of *DLX-3*, *DLX-4* (B) and *TOP2A* genes (C)



6. PhD thesis

Funding of this project allowed us to host a graduate student, Ms. Savana Santos, from the Federal University of Parana in Curitiba, Brazil, to complete the practical part of her PhD thesis in our Laboratory. Ms Santos' work on this project will be a major component of her PhD thesis which she will defend next year (2007). Letter from her mentors is included in the appendix.

7. Grants submitted using preliminary data from this project

The data generated in this project was used as preliminary data to support 3 grants currently under review, to fund a larger study:

-Prediction of Breast Cancer Recurrence in Lymph Node Negative Patients
American Cancer Society- Career Development Award April 1st, 2006 (PI: L. Cavalli)

-Prediction of Breast Cancer Recurrence in Lymph Node Negative Patients
NIH-R21- April 2006 (PI: L. Cavalli)

-Prediction of recurrence in patients with early stage breast cancer
Komen – August 2006 (PI: L. Cavalli)

KEY RESEARCH ACCOMPLISHMENTS

- Accrual of thirty patients with invasive breast cancer and SLN involvement at the time of diagnosis and known clinical information and outcome.
- Pathology review of all the samples collected
- Cellular microdissection and DNA isolation of tumor specimens
- Successful CGH analysis of twenty tumor pairs (Primary tumors and corresponding SLN metastatic lesions) and comparison of the findings between each pair and across the two groups.
- Identification of a subset of frequent genetic alterations commonly observed in both groups, occurring in 30-60% of the cases.
- Identification of a subset of genetic alterations more commonly detected in either the SLN lesions or the primary tumors
- Optimization of a DNA isolation method for oligo-array analysis of FFPE samples.
- Optimization of a DNA labeling method for oligo-array analysis of FFPE samples.
- Optimization of an oligo array CGH protocol for analysis of FFPE samples.

REPORTABLE OUTCOMES

1- Platform Presentation at an International Scientific Meeting:

“Genomic analysis of sentinel lymph nodes: in search of predictive markers of breast cancer metastasis”.

Bassem R. Haddad, Savana L. Santos, Enilze M. Ribeiro, Janice D. Rone, Cicero A. Urban, Rubens S. Lima, Iglenir J. Cavalli, **Luciane R. Cavalli**.

Presented as a PLATFORM at the 56th Annual Meeting of the American Society of Human Genetics (ASHG). New Orleans, LA, October 9th – 13th, 2006.

Abstracts presented at scientific meetings (see appendices)

2- Poster Presentations at National and International Scientific Meetings:

1.“Detection of genetic alterations in breast sentinel lymph nodes by CGH.”

Cavalli LR, Santos SL, Urban CA, Lima RS, Cavalli IJ, Haddad BR.

Presented at the Era of Hope Meeting- DOD Meeting, Philadelphia, PA, June 8-11, 2005.

2.“DNA copy number changes in breast sentinel lymph node metastasis.”

Cavalli LR, Santos SL, Ribeiro E, Urban CA, Lima RS, Cavalli IJ, Haddad BR.

Presented at the American Association for Cancer Research (AACR) Meeting - Frontiers in Cancer Prevention Research Meeting - Baltimore, MD, October 30th - November 2nd, 2005.

3. "Comparison between genomic alterations of primary breast tumors and their corresponding sentinel lymph node metastatic lesions".

Savana L. Santos, Iglenir J. Cavalli, Enilze M. Ribeiro, Cicero A. Urban, Rubens S. Lima, Bassem R. Haddad, **Luciane R. Cavalli**.

Presented at the 97th Annual Meeting of American Association for Cancer Research (AACR) Washington DC, April 1st-5th, 2006.

4. "DNA copy number changes associated with Breast sentinel lymph node metastasis".

Luciane R. Cavalli, Savana L. Santos, Iglenir J. Cavalli, Enilze M. Ribeiro, Cicero A. Urban, Rubens S. Lima, Bassem R. Haddad.

Presented at the "50 years of 46 Human Chromosomes: Progress in Cytogenetics" conference: NCI/NIH, Bethesda, MD. July 20-21, 2006.

3- PhD Thesis:

Work supported by this grant is a main component of Ms. Savana Santos PhD Thesis. (will be defended in 2007).

CONCLUSIONS

This study supported by a concept award (W81XWH04-1-0671) demonstrates the utility of CGH analysis of tumors as a tool to detect changes that may be associated with, and predispose to, metastasis of primary breast tumors. Based on the number of cases studied, a set of chromosomal changes common to both the primary and the SLN metastatic lesions was identified and a set of changes preferentially present in either lesions was also identified. Our initial findings justify the initiation of a larger study. Towards that end, the PI submitted 3 new grants (currently under review) using the data generated in this concept award as preliminary data. This project lead to the optimization of a protocol allowing the evaluation of FFPE tumor specimens by oligo array CGH. The implication of such analysis is tremendous: it allows the refinement of CGH findings and the detection of alterations of specific genes.

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APPENDICES

Letter from Savana Santos' PhD methors:



Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Departamento de Genética

Elaine Sávio Cavalli, PhD
Federal University of Paraná
Department of Genetics
Hematology and Genetics Laboratory
Curitiba, Paraná, Brazil

March 17, 2006

Laciene R Cavalli, PhD
Georgetown University Medical Center
Lombardi Comprehensive Cancer Center
Department of Oncology

Dear Dr. Cavalli

I would like to thank you for having provided the opportunity to Ms. Savana Santos, one of our graduate students at the Federal University of Paraná in Curitiba, Brazil, to spend one year in your laboratory at Georgetown University Medical Center, between March 2005 and February 2006, in order to complete the practical/experimental part of her PhD thesis.

During her stay in your laboratory, Ms. Santos gained valuable research experience both at the practical, and theoretical levels. Her work in your laboratory focused on studying genetic abnormalities in the sentinel lymph nodes using Comparative Genomic Hybridization (CGH). We were very impressed with the data she generated during her stay with your group; these data will be summarized in a manuscript and will constitute a significant component of her Doctorate thesis. Her PhD dissertation proposal received the approval of her thesis committee and will be defended during 2007.

Ms. Santos was able to acquire the necessary skills to conduct state-of-the-art cancer research studies, and is in the process of implementing several molecular methodologies in our laboratory in Brazil, such as CGH and other methods, that she learned during her stay with your group.

While Ms. Santos' stay in your lab was supported through a fellowship award she received from the National Council of Research and Technology (CNPq) in Brazil (a national agency supporting research), we understand that the project she worked on was supported by a Research grant that you received from the US Department of Defense (DOD). This funding allowed your research project to take place and gave the opportunity to our student to train in your lab. Ms. Santos' excellent experience with your research group, contributed to her personal and professional growth and scientific advance, and strengthened the interactions between our research groups.

We are much appreciative of this collaboration and are looking forward to further interactions.

Sincerely,
Elaine Sávio Cavalli (mentor)
Erica D. Leal
Laizte M. Ribeiro (co-mentor)

Abstracts presented at scientific meetings:

1. Presented at the Era of Hope Meeting- Philadelphia, PA, June 8-11, 2005.
2. Presented at the AACR - Frontiers in Cancer Prevention Research Meeting Baltimore, MD, October 30th - November 2nd, 2005.
3. Presented at the 97th Annual Meeting of American Association for Cancer Research (AACR) Washington DC, April 1st-5th, 2006.
4. Presented at the "50 years of 46 Human Chromosomes: Progress in Cytogenetics" conference: NCI/NIH , Bethesda, MD. July 20-21, 2006.
5. Presented as an oral platform at the 56th Annual Meeting of the American Society of Human Genetics (ASHG). New Orleans, LA, September 9th – 13th , 2006.

1. "Detection of genetic alterations in breast sentinel lymph nodes by CGH."

LR Cavalli, SL Santos, CA Urban, Lima RS, BR Haddad

Background: Genome-wide based methodologies can reveal genes or chromosomal regions specifically altered during the process of breast cancer progression. Genetic studies comparing changes in distant metastatic lesions with those found in the corresponding primary tumors have revealed different alterations between these lesions, suggesting that specific events may be associated with metastatic dissemination. The genetic analysis of paired samples has made it possible to assess the degree of clonal divergence and genetic heterogeneity that characterize the metastatic process. In breast cancer, axillary lymph node status remains the single most important prognostic variable and a crucial component to the staging system. The sentinel lymph node (SN) is the first node to harbor malignant cells in breast tumors with metastases, and SN positivity is an indication for axillary lymph node dissection.

Purpose/Rationale: The purpose of this study is to identify specific genetic alterations in the metastatic sentinel lymph node lesions, in comparison to the ones observed in the corresponding primary tumors from patients with breast cancer using Comparative Genomic Hybridization (CGH). The characterization of genetic alterations at the SN site is a logical step to define the cytogenetic evolution of primary tumors to a metastatic state, and may represent the initial genetic events that occur in the early metastatic process.

Methods: The tissue samples are obtained from paraffin embedded archival blocks. Prior to the CGH analysis, after a histological evaluation, sections of the primary and the metastatic tumor tissue are microdissected using a modified razor blade to ensure minimal contamination of normal or stromal cells. The DNA is extracted from these sections (average 5 slides with 5mM sections per sample) and labeled by nick-translation. CGH followed previously described protocol.

Results: Eight pairs of primary tumors and SN metastatic lesions were analyzed by conventional CGH. Chromosomal abnormalities were observed in all the cases. The most frequent gains and loss observed were: -13q13-q32, +6q13-q23, +11p15-q21, +12q23-ter +16, +20. Loss on 6q and gain of chromosome 20 were more frequently observed in the SLN group, whereas gain on 12q and 20q were more frequent seen in the primary tumors. Gain on 6p, observed in 33.3% of the cases was only observed in the SN group. The 13q loss, the most common abnormality in this study, was equally observed in both groups.

Future: We plan to study total number of 30 paired samples using CGH.

Relevance: The identification of genetic alterations present in the SN of the breast will be important to detect the early genetic alterations that occur in the metastatic process. Ultimately these alterations can be used as additional molecular markers, that can help in the reduction or elimination of the need for invasive surgical procedures.

Acknowledgments: "The US Army Medical Research and Materiel Command under W81XWH-04-1-0671 supported this work.

2. DNA copy number changes in breast sentinel lymph node metastasis.

Luciane R. Cavalli,¹ Savana L. Santos,² Enilze M. Ribeiro,³ Cicero A. Urban,⁴ Rubens S. de Lima,⁴ Iglenir J. Cavalli,³ Bassem R. Haddad,¹ Georgetown University Medical Center,¹ Washington, DC, Georgetown University Medical Center and Federal University of Parana,² Washington, DC, Federal Univ. of Parana, Genetics Dept.,³ Curitiba, Parana, Brazil, Surgery Service - Hospital Nossa Sra. das Gracas,⁴ Curitiba, Parana, Brazil.

The sentinel lymph node (SLN) is the first node in the axilla to harbor malignant cells in breast tumors with metastasis, and its positivity is an indication for axillary lymph node dissection. Characterization of genetic alterations in the SLN metastatic lesions is a logical step for better defining the evolution of primary tumors to a metastatic state, and may represent the initial genetic events that occur early in the metastatic process, before distant metastasis takes place. Several studies have been performed to "profile" breast tumors and their metastatic lesions in the distant axillary lymph nodes, but none has looked at metastasis in SLN. In addition, very few of these studies have been performed in paired primary tumors and metastatic lesions from the same patient. Here we describe the results of DNA copy number changes observed in paired primary tumors and their corresponding metastatic sentinel lymph node lesions using Comparative Genomic Hybridization (CGH) analysis. The tissue blocks were obtained from the Hospital Nossa Senhora das Gracas, Brazil. CGH was performed using tumor DNA obtained from malignant cells isolated by microdissection from paraffin embedded archival material. A total of ten pairs of primary tumors and SLN metastatic lesions were analyzed. Chromosomal abnormalities were observed in all the cases. The most frequent gains (+) and losses (-) observed were: -1p31~p21, +17, +19 and +20. These alterations were observed in both groups. Gain on chromosome 20 was the most frequently observed in the primary tumor group, whereas losses on 1p31~p21 and gains on chromosomes 17 and 19 were equally observed in both groups. An additional number of paired samples of primary tumors and corresponding SLN metastatic lesions are currently being evaluated. This study will allow the assessment of the degree of clonal divergence and genetic heterogeneity that characterize the metastatic process. Identification of genetic alterations present in the SLN of the breast will be important to detect early genetic alterations that occur in the metastatic process. Ultimately these alterations can potentially be used as additional molecular markers that can help in the reduction or elimination of the need for invasive surgical procedures, such as axillary lymph node dissection.

"The US Army Medical Research and Materiel Command under W81XWH-04-1-0671 supported this work.

3. Comparison between genomic alterations of primary breast tumors and their corresponding sentinel lymph node metastatic lesions

Savana L. Santos, Iglenir J. Cavalli, Enilze M. Ribeiro, Cicero A. Urban, Rubens S. Lima, Bassem R. Haddad, Luciane R. Cavalli. Federal University of Parana, Curitiba, Brazil, Federal University of Parana, Curitiba, Brazil, Hospital Nossa Senhora das Gracas, Curitiba, Brazil, Georgetown Univ. Medical Ctr., Washington, DC

The sentinel lymph node (SLN) is the first node in the axilla to harbor malignant cells in breast tumors with metastasis, and its positivity is an indication for axillary lymph node dissection. Characterization of genetic alterations in the SLN metastatic lesions is a logical step for better defining the evolution of primary tumors to a metastatic state, and may represent the initial genetic event that occur early in the metastatic process, before distant metastasis takes place. Several studies have been performed to "profile" breast tumors and their metastatic lesions in distant axillary lymph nodes, but none has looked at metastasis in SLN. In addition, very few of these studies have been performed in paired primary tumors and metastatic lesions from the same patient. In an effort to precisely characterize early genomic changes that are associated with and may predispose to metastasis, we initiated this study to compare genomic alterations between primary breast tumors and their corresponding sentinel lymph node metastatic lesions using comparative genomic hybridization (CGH). Here, we report our results from the analysis of the 1st ten cases (paired samples). The tissue blocks were obtained from the Hospital Nossa Senhora das Gracas, Brazil. CGH was performed using tumor DNA obtained from malignant cells isolated by microdissection from paraffin embedded archival material. Chromosomal abnormalities were observed in all 10 cases studied, in the primary lesions as well as in the SLN metastasis. The most frequent gains (+) and losses (-) observed were: -1p31~p21, +17, +19 and +20. These alterations were observed in both groups. Gain on chromosome 20 was the most frequently observed in the primary tumors group, whereas losses on 1p31~p21 and gains on chromosomes 17 and 19 were equally observed in both groups. An additional number of paired samples of primary tumors and corresponding SLN metastatic lesions are currently being evaluated. This study will allow the assessment of the degree of clonal divergence and genetic heterogeneity that characterize the metastatic process. Identification of genetic alterations present in the SLN of the breast will be important to detect early genetic alterations that occur in the metastatic process. These alterations can potentially be used as additional prognostic markers that can help in the reduction or elimination of the need for invasive surgical procedures, such as axillary lymph node dissection, to predict outcome.

Acknowledgments: "The US Army Medical Research and Materiel Command under W81XWH-04-1-0671 supported this work.

4. DNA copy number changes associated with breast sentinel lymph node metastasis

Luciane R. Cavalli, Savana L. Santos, Enilze M. Ribeiro, Janice D. Rone, Cicero A. Urban, Rubens S. Lima, Iglenir J. Cavalli, Bassem R. Haddad.

Metastases in axillary lymph nodes confer a high risk for recurrence and are the most important predictor of prognosis in early breast cancer. The sentinel lymph node (SLN) is the first node in the axilla to harbor malignant cells in breast tumors with metastasis, and its positivity is an indication for axillary lymph node dissection. Characterization of genetic alterations in the SLN metastatic lesions is a logical step for better defining the evolution of primary tumors to a metastatic state, and may represent the initial genetic event that occur early in the metastatic process, before distant metastasis takes place. Several studies have been performed to "profile" breast tumors and their metastatic lesions in distant axillary lymph nodes, but none has looked at metastasis in SLN. In addition, very few of these studies have been performed in paired primary tumors and metastatic lesions from the same patient. In an effort to precisely characterize early genomic changes that are associated with and may predispose to metastasis, we initiated this study to compare genomic alterations between primary breast tumors and their corresponding sentinel lymph node metastatic lesions using comparative genomic hybridization (CGH). Here, we report our results from the analysis of the 1st ten cases (paired samples). The tissue blocks were obtained from the Hospital Nossa Senhora das Gracas, Brazil. CGH was performed using tumor DNA obtained from malignant cells isolated by microdissection from paraffin embedded archival material. Chromosomal abnormalities were observed in all 10 cases studied, in the primary lesions as well as in the SLN metastasis. The most frequent gains (+) and losses (-) observed were: -1p31~p21, +17, +19 and +20. These alterations were observed in both groups. Gain on chromosome 20 was the most frequently observed in the primary tumors group, whereas losses on 1p31~p21 and gains on chromosomes 17 and 19 were equally observed in both groups. An additional number of paired samples of primary tumors and corresponding SLN metastatic lesions are currently being evaluated. This study will allow the assessment of the degree of clonal divergence and genetic heterogeneity that characterize the metastatic process. Identification of genetic alterations present in the SLN of the breast will be important to detect early genetic alterations that occur in the metastatic process. These alterations can potentially be used as additional prognostic markers that can help in the reduction or elimination of the need for invasive surgical procedures, such as axillary lymph node dissection, to predict outcome. "The US Army Medical Research and Materiel Command under W81XWH-04-1-0671 supported this work.

5. Genomic analysis of sentinel lymph nodes: in search of predictive markers of breast cancer metastasis

Bassem R. Haddad, Savana L. Santos, Enilze M. Ribeiro, Janice D. Rone, Cieero A. Urban, Rubens S. Lima, Iglenir J. Cavalli, Luciane R. Cavalli. Georgetown Univ. Medical Ctr., Washington, DC, Federal University of Parana, Curitiba, Brazil, Hospital Nossa Senhora das Gracas, Curitiba, Brazil

Despite significant improvement in our understanding of the mechanisms of breast cancer development and progression, accurate prediction of the potential for future metastasis of non-metastatic primary tumors remains illusive. We set out to determine new predictive markers to augment the current criteria used to assess prognosis. The sentinel lymph node (SLN) is the first node to harbor malignant cells in breast tumors with metastasis. Because genetic alterations in the SLN lesions are likely to represent early and significant changes in the process of metastasis, characterization of such changes may aid in the discovery of new prognostic markers. Towards this end, we initiated this study to compare genomic alterations between primary breast tumors and their corresponding sentinel lymph node metastatic lesions using CGH analysis. Here, we report our results from the analysis of the 1st fourteen cases (paired samples). Although the number of cases analyzed so far is too small for statistical analysis, an interesting trend emerged: i) chromosomal abnormalities were observed in all 14 cases studied in both the primary tumors and the SLN lesions; ii) despite the diversity in the alterations detected in each case, both the primary and metastatic lesions shared a small number of alterations [gains (+) and losses (-)], specifically: -1p31~p21, +17, +19 and +20; iii) the above four alterations were also the most commonly observed in the whole set; iv) gain on chromosome 20 was more frequently observed in the primary tumors, whereas losses on 1p31~p21 and gains on 17 and 19 were equally observed in both groups. This study will allow assessment of clonal divergence and genetic heterogeneity that characterize the metastatic process and identification of a subset of relevant genetic alterations associated with metastasis. These can potentially be used as additional markers to predict metastasis thus reducing the need for invasive surgical procedures (e.g. axillary lymph node dissection). The US Army Medical Research and Materiel Command under W81XWH-04-1-0671 supported this work.